APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1320–1329
0099-2240/82/061320-10$02.00/0

Vol. 43, No. 6

Fluorogenic Assays for Immediate Confirmation of *Escherichia coli*†

PETER C. S. FENG‡ AND PAUL A. HARTMAN*

Department of Microbiology, Iowa State University, Ames, Iowa 50011

Received 9 October 1981/Accepted 8 March 1982

Rapid assays for *Escherichia coli* were developed by using the compound 4-methylumbelliferone glucuronide (MUG), which is hydrolyzed by glucuronidase to yield a fluorogenic product. The production of glucuronidase was limited to strains of *E. coli* and some *Salmonella* and *Shigella* strains in the family *Enterobacteriaceae*. For immediate confirmation of the presence of *E. coli* in most-probable-number tubes, MUG was incorporated into lauryl tryptose broth at a final concentration of 100 μg/ml. Results of both the presumptive test (gas production) and the confirmed test (fluorescence) for *E. coli* were obtained from a variety of food, water, and milk samples after incubation for only 24 h at 35°C. Approximately 90% of the tubes showing both gas production and fluorescence contained fecal coliforms (they were positive in EC broth incubated at 45°C). Few false-positive reactions were observed. The lauryl tryptose broth–MUG–most-probable-number assay was superior to violet red bile agar for the detection of heat- and chlorine-injured *E. coli* cells. Anaerogenic strains produced positive reactions, and small numbers of *E. coli* could be detected in the presence of large numbers of competing bacteria. The fluorogenic assay was sensitive and rapid; the presence of one viable cell was detected within 20 h. *E. coli* colonies could be distinguished from other coliforms on membrane filters and plates of violet red bile agar if MUG was incorporated into the culture media. A rapid confirmatory test for *E. coli* that is amenable to automation was developed by using microtitration plates filled with a nonselective medium containing MUG. Pure or mixed cultures containing *E. coli* produced fluorescence within 4 h (most strains) to 24 h (a few weakly positive strains).

The detection and enumeration of indicator bacteria are of primary importance for monitoring sanitation and the microbiological quality of food and water. Coliforms, fecal coliforms, and *Escherichia coli* are all used as indicators of fecal pollution. Among these, *E. coli* is often preferred as an indicator because it is specific and most reliably reflects fecal origin.

In the United States, the two accepted methods for the analysis of coliforms in water and wastewater are the membrane filtration technique with mFC broth incubated at 44.5 ± 0.2°C and the most-probable-number (MPN) method, which involves a presumptive test followed by a confirmed test (3). In food and dairy product analysis, the MPN assay is also accepted, as is a direct plating procedure with violet red bile (VRB) agar (2). These assays are based on the properties of acid or gas production from the fermentation of lactose, followed by confirmatory tests. These tests are laborious and time-consuming; some require 96 h to complete.

E. coli detection methodologies have been reported to be inadequate, especially when injured cells are present (11, 14, 29, 37, 39). Debilitated *E. coli* cells are sensitive to high incubation temperatures (34, 40) and to selective media used for their detection and enumeration (31, 39). In addition, the MPN assay is susceptible to bacterial interference (1, 18, 25); false-negative reactions (absence of gas production in the presence of coliforms) may occur at the presumptive, confirmed, and completed stages of the MPN analysis (13). Other factors, such as synergistic gas production from lactose by noncoliforms (20), cultivation of anaerogenic and non-lactose-fermenting *E. coli* strains (4), and the presence of lactose-fermenting noncoliforms (23, 24), have contributed to the inefficiency of *E. coli* detection methodologies.

The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to existing methods. Enzymatic reactions are specific, rapid, and sensitive. For example, approximately 97% of the *E. coli* strains examined by

† Journal paper no. J-10415 of the Iowa Agriculture and Home Economics Experiment Station, Ames; project no. 2377.

‡ Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

Kilian and Bülow (26) produced β-glucuronidase (GUD) (H. J. Buehler, P. A. Katzman and E. A. Doisy, Fed. Proc. 8:189, 1949); almost all other *Enterobacteriaceae* organisms lacked the enzyme (26).

GUD activities were initially detected with chromogenic substrates (15); however, a more sensitive assay with the fluorogenic compound, 4-methylumbelliferone glucuronide (MUG) has been used in recent years (10, 30, 33). GUD cleaves MUG to release a fluorogenic end product that is visible under long-wave UV light. The present study was undertaken to develop rapid, specific, and more efficient detection assays for *E. coli* by using the fluorogenic substrate MUG to detect GUD-positive bacteria.

MATERIALS AND METHODS

Stock cultures and media. Most of the bacterial strains were obtained from collections maintained at Iowa State University, Ames. All media were commercial products obtained from Difco Laboratories, Detroit, Mich. The substrate, MUG, was purchased from Sigma Chemical Co., St. Louis, Mo. MUG is practically insoluble in cold water; therefore, it was dissolved in warm water and then filtered through a 0.22-μm membrane or heated to 121°C for 15 min for sterilization.

Specificity studies. The presence of GUD was determined by using a medium developed by Dahlén and Linde (10). It contained 20 g of tryptose, 5 g of NaCl, 1 g of cysteine hydrochloride, and 15 g of Noble agar per liter. MUG was used at a final concentration of 100 μg/ml; it was sterilized by filtration and was added after the basal medium had been sterilized and cooled. The tempered agar-substrate was dispensed into the wells of microtitration plates. To assay for GUD activity, growth from overnight cultures was stabbed into individual wells, and the plate was sealed with microtiter plate tape to prevent cross contamination between wells. The plate was incubated overnight at 35°C and examined for the appearance of fluorescence under long-wave UV light (Blacklight blue, Westinghouse [Bloomfield, N.J.]; emission, about 366 nm). Because of their short wavelength, most germicidal lamps are not suitable for this purpose.

Applications of MUG. The possibilities of using MUG directly in conventional coliform media were examined by using three coliform detection methods. All media were prepared as specified by the manufacturers.

With the membrane filter-mEndo broth technique, 100 μg of MUG per ml was incorporated into the selective broth before the medium was dispensed onto sterile paper pads. A membrane filter containing a mixed inoculum of *E. coli* and *Enterobacter aerogenes* was incubated on top of the medium-saturated pad. Unless otherwise noted, the time and temperature of incubation conformed to *Standard Methods for the Examination of Water and Wastewater* (3).

The application of MUG in a direct plating procedure was examined by using the VRB-2 agar overlay method (21). Overnight cultures of *E. coli* and *E. aerogenes* were mixed and diluted in sterile 9-ml blanks of phosphate-buffered saline (PBS; 8.5 g of NaCl, 0.39 g of $NaH_2PO_4 \cdot H_2O$, 1.0 g of Na_2HPO_4 per liter [pH 7.2]). Portions (1 ml each) were plated in 10 ml of molten, tempered tryptic soy agar (TSA), and the plates were incubated at 35°C for 2 h. After preenrichment, the base agar was overlaid with 10 ml of VRB-2 agar, which contained 200 μg of MUG per ml.

In the MPN method, MUG was incorporated into the presumptive medium. Appropriate concentrations of MUG were dissolved in warm water and mixed with lauryl tryptose broth (LTB) to obtain a final substrate concentration of 100 μg/ml. The LTB-MUG medium was then dispensed into tubes containing Durham vials, and the tubes were sterilized in an autoclave. Comparisons of LTB-MUG medium and normal LTB medium were made by inoculating duplicate sets of each medium with 0.1 ml of an overnight broth culture of *E. coli*.

Analysis of naturally contaminated samples. The efficiency of the LTB-MUG medium in the detection of *E. coli* was examined by using the five-tube MPN method.

Raw surface water and wastewater effluents were collected from nearby rivers and streams and from the Ames Wastewater Treatment Plant. Water samples were diluted to extinction in 9-ml PBS blanks, and 0.1-ml portions of appropriate dilutions were used to inoculate LTB-MUG medium. Each assay was set up in triplicate (15 tubes per dilution) and incubated as specified previously (3). Presumptive counts of coliforms (gas production) and *E. coli* (fluorescence) were determined. All tubes that were gas positive [gas (+)], fluorescence positive [fluorescence (+)], or both were subcultured into EC broth and incubated at 45°C to confirm the presence of fecal coliforms. A comparative fecal coliform count for each water sample was also obtained by using the standard membrane filter-mFC broth method, except that incubation was at 45°C. The total viable bacterial content of each sample was obtained by surface plating on tryptone glucose extract agar (TGEA) plates.

Ground beef, chicken giblets, and frozen broccoli were purchased from local retail markets. Raw milk samples were collected from the Iowa State University Dairy Farm, Ames. Three separate samples of milk and of each food category were analyzed, in triplicate, by using the five-tube MPN assay with LTB-MUG medium.

The food samples were prepared as described in *Compendium of Methods for the Microbiological Examination of Foods* (2). Each 25-g sample was homogenized at high speed for 2 min with 225 ml of 0.1% peptone water in a Waring blender. After a homogenate was allowed to settle for 2 min, each sample was diluted to extinction in PBS, and 0.1-ml samples from each dilution were inoculated into LTB-MUG tubes. Raw milk samples were mixed thoroughly, serially diluted to extinction in PBS, and analyzed as described for food samples. All tubes were incubated under conditions specified previously (2), and all tubes that were gas (+), fluorescence (+), or both were subcultured into EC broth medium at 45°C to confirm the presence of fecal coliforms.

Sensitivity studies. An overnight culture of *E. coli* B cells grown in LTB was serially diluted to extinction in PBS blanks. Samples 0.1 ml from each dilution were then inoculated into LTB-MUG tubes and plated on TSA plates to determine the number of viable cells

present. Inoculated tubes were incubated at 35°C and monitored closely for the onset of fluorescence. The sensitivity was determined by correlating the number of cells initially present (as determined by plate counts on TSA) versus the approximate time at which fluorescence was detected. The lowest detection limit was assessed by the least number of cells at the time that fluorescence appeared.

Detection of injured *E. coli* cells. The efficiency of LTB-MUG medium in relation to VRB-2 agar for the detection of injured bacteria was examined with heat- and chlorine-injured cells.

An overnight culture of *E. coli* B cells grown in tryptic soy broth was adjusted to the density of a McFarland standard no. 3, and the initial concentration of cells was determined by plate counts on TSA plates. The cell suspension was then placed in a 56°C water bath and incubated for 10 min. The number of viable cells present after treatment was determined by using TSA plates and VRB-2 agar medium. The fraction of cells that grew on TSA but not on VRB-2 was considered to be severely injured. The heat-treated sample was also diluted to extinction in PBS and inoculated into LTB-MUG tubes for a five-tube MPN analysis. All samples were incubated at 35°C for 24 to 48 h.

The procedure described by Camper and McFeters (8) was used to injure *E. coli* cells with chlorine. A 12- to 16-h culture of *E. coli* B was grown in 100 ml of tryptic soy broth and harvested by centrifugation. The cells were washed several times in PBS and resuspended in 1,000 ml of chlorine-free water. The initial concentration of cells was determined by plating on TGEA and VRB-2 agar. A chlorine solution was prepared by diluting 1 ml of Clorox bleach (5.25% sodium hypochlorite) in 99 ml of chlorine-free water to obtain a stock solution of 500 mg of chlorine per liter; one ml of this stock solution was then added to 1,000 ml of cell suspension, resulting in a final chlorine concentration of 0.5 mg/liter. The suspension was agitated at room temperature for 8 min. Samples (1 ml each) were then removed, mixed with 1 ml of 0.1 N sodium thiosulfate, diluted in PBS, and plated on TGEA and VRB-2 agar to assess the fraction of injured cells. The samples were also diluted to extinction and inoculated into LTB-MUG tubes for a five-tube MPN analysis. All plates and tubes were incubated at 35°C for 24 to 48 h. In the LTB-MUG-MPN assay, each sample was examined for both fluorescence and gas production.

Bacterial interference. A study was conducted to examine whether false-negative MPN reactions (absence of gas production in the presence of *E. coli*) due to coliform suppression may be detected by the appearance of fluorescence in LTB-MUG medium.

A preliminary study showed that *Proteus vulgaris* was able to suppress gas production by *E. coli* when these organisms were simultaneously inoculated into LTB tubes. A culture of *P. vulgaris* was grown overnight in a tube of LTB-MUG medium to make certain that it did not possess GUD activity or produce gas from the fermentation of lactose. The suspension was then serially diluted to obtain various concentrations of cells. An LTB culture of *E. coli* was also diluted in PBS, and various concentrations of *E. coli* and the antagonist were added to LTB-MUG tubes. The number of cells present at each dilution was determined by plate counts on TGEA. The inoculated tubes were incubated at 35°C and observed for fluorescence and gas production.

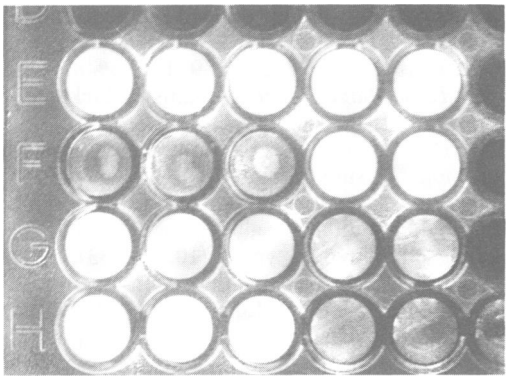

FIG. 1. Microtitration plate-MUG assay for bacterial GUD activity. Row G contains (left to right) one strongly positive culture, two weakly positive cultures, two negative cultures, and an empty well.

Rapid enumeration assay. A rapid fluorogenic assay for *E. coli* was tested. Wells in a microtitration plate were filled with TSA containing 100 μg of MUG per ml. The wells were arranged in nine rows of five wells each, so that each set represented triplicate determinations of a five-well MPN analysis. Samples of sewage effluent were serially diluted to extinction in PBS blanks; 1 drop (0.05 ml) of appropriate dilutions was inoculated into each well. The microtitration plate was sealed with microtiter plate tape and incubated at 35°C. Wells that fluoresced were counted and used to obtain MPN estimates.

RESULTS

Specificity of bacterial GUD. The presence of GUD in gram-negative bacteria was examined by using a microtitration plate assay (Fig. 1). Among 198 strains tested (Table 1), GUD activity was present only in random isolates identified as *E. coli* (96%), 10 strains of enterotoxigenic *E. coli* (100%), *Salmonella* spp. (17%), and *Shigella*

TABLE 1. Presence or absence of GUD activity in 198 strains of gram-negative bacteria

Organism	No. tested	No. positive (%)
Citrobacter spp.	4	0 (0)
Enterobacter spp.	9	0 (0)
E. coli	110	106 (96)
Enterotoxigenic *E. coli*	10	10 (100)
Klebsiella spp.	11	0 (0)
Proteus spp.	4	0 (0)
Pseudomonas spp.	8	0 (0)
Salmonella spp.	35	6 (17)
Serratia spp.	2	0 (0)
Shigella spp.	5	2 (40)

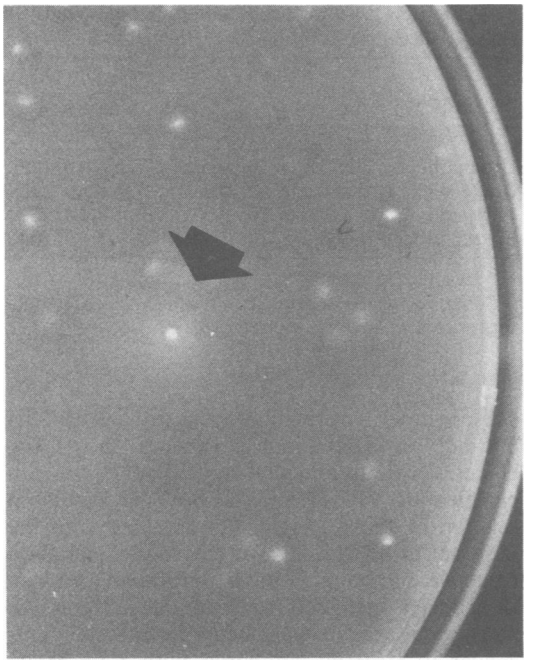

FIG. 2. Photograph of a VRB agar plate showing an *E. coli* colony (with halo, arrow) and numerous GUD-negative colonies.

spp. (40%); all other genera tested were negative. The enzymatic reaction was rapid: most reactions occurred within 4 h; however, some weakly GUD-positive strains required overnight (16-h) incubation.

Application of MUG in conventional coliform assays. With the membrane filter-mEndo broth technique, the presence of MUG efficiently distinguished fecal and nonfecal coliforms. *E. coli* colonies, which appeared red like any coliform on mEndo broth, fluoresced when exposed to UV light. *E. aerogenes* colonies were nonfluorogenic (data not shown). In the direct plating method with VRB-2 agar, *E. coli* colonies were differentiated from other coliforms by the appearance of fluorescent halos (Fig. 2). In both methods, the reactions were rapid; fluorescence was usually detectable within 12 to 20 h of incubation. The results were observed within 24 h, however, because the 4-methylumbelliferone released by GUD activity diffused into the surrounding medium and eventually covered the entire surface area.

When MUG was incorporated into LTB, the presence of *E. coli* was detected by the appearance of fluorescence throughout the entire tube. *E. coli* produced gas in equivalent amounts when inoculated into LTB-MUG and normal LTB medium (Fig. 3). However, the LTB-MUG medium fluoresced when examined under UV light, confirming immediately that *E. coli* was present. Similarly, when the two tubes to the left side of Fig. 3 were inoculated with an anaerogenic strain of *E. coli*, gas was not produced, but fluorescence appeared in the tube of LTB-MUG medium.

Detection of *E. coli* in food and water. The geometric means of bacterial counts and the percent recoveries from 10 water samples were calculated. When the mean (1.12×10^5/ml) obtained for gas production in LTB was established as 100%, the fraction of *E. coli* present (fluorescence) was 1.14×10^4, or approximately 10%. The fraction of fecal coliforms detected by the mFC broth method (9.8×10^3/ml) was 8.7%. All LTB-MUG tubes that showed gas (+), fluorescence (+), or both were subcultured into EC broth and incubated at 45°C to check for the presence of fecal coliforms (Table 2). Approximately 85% of the gas (+) fluorescence (+) tubes contained fecal coliforms, but there also

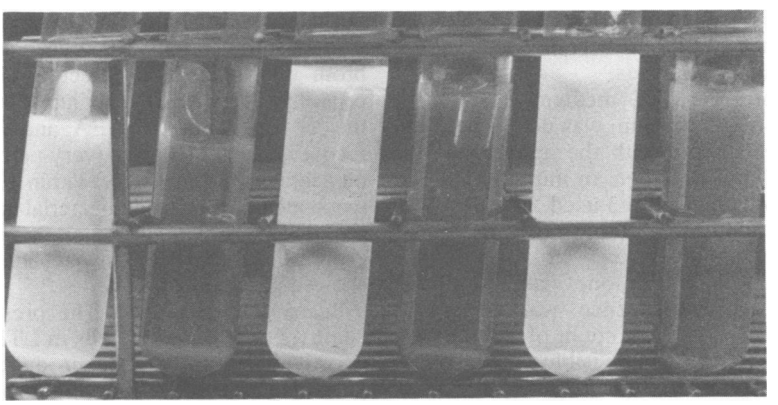

FIG. 3. Three sets of LTB-MUG (fluorescent) and LTB (nonfluorescent) tubes inoculated with *E. coli* and photographed under UV light after incubation. The pair of tubes on the left contained an anaerogenic strain of *E. coli*.

TABLE 2. Results of EC broth fecal coliform confirmatory tests on LTB-MUG tubes obtained from the MPN analysis of water samples

LTB-MUG reaction	No. tested	Positive EC broth test (%)
Gas (+) fluorescence (+)	72	61 (85)
Gas (+) fluorescence (−)	72	7 (9)
Gas (−) fluorescence (+)	17	0 (0)

was a 9% apparent false-positive rate (production of gas in EC medium in the absence of fluorescence). In addition, 17 gas (−) fluorescence (+) tubes failed to produce gas in the EC confirmatory test (apparent false-negatives). We were able, however, to isolate the fluorescence-causing organisms in 4 of these 17 tubes by making streak plates on eosin methylene blue agar and TGEA. By using the Minitek identification system (BBL Microbiology Systems, Cockeysville, Md.), two of the four isolates were identified as non-gas-forming strains of *E. coli* because they were identified as *E. coli* by Minitek tests but failed to produce gas when subcultured back into LTB tubes. Of the other two isolates, one was a *Salmonella* sp. and the other was a *Shigella* sp., which would account for the lack of gas production from lactose.

Table 3 shows the results of food and milk analyses by using the LTB-MUG-MPN assay. Each count was the mean of three replicates per food category, and the percentages containing *E. coli* (fluorescence) were determined in relation to total coliforms (gas production). The highest percentage of *E. coli* (28%) was present in meat samples.

The EC broth confirmatory test results for food and dairy samples are shown in Table 4. A total confirmatory rate of 91% was obtained for all gas (+) fluorescence (+) tubes. A low false-positive rate of 2% was also observed (positive EC test in the absence of fluorescence). No gas (−) fluorescence (+) tubes were encountered in the food and dairy analyses.

Sensitivity of LTB-MUG medium. The sensitivity of LTB-MUG medium was determined by correlating cell counts with the appearance of fluorescence (Fig. 4). When an initial inoculum of about 10^4 cells per ml was used, fluorescence was observed in 4 h. Detectable gas evolution at this same inoculum level was not observed until approximately 9 h. If only one viable cell was present initially, fluorescence was observed within 20 h. The viable cell count of *E. coli* was 10^7 to 10^8 cells per ml when visible fluorescence first appeared at 35°C.

Recovery of injured cells. Heat treatment at 56°C for 10 min caused a 94% reduction in the number of viable *E. coli* cells. Among the survivors, over 99% were injured; these grew on TSA but not on VRB-2 agar. Table 5 shows the bacterial counts and the percent recovery efficiencies of VRB-2 and LTB-MUG media in relation to TSA. The results obtained with the LTB-MUG-MPN assay were based only on fluorescence, although all tubes that fluoresced also showed gas production. A significantly lower mean recovery rate was observed with the VRB-2 agar (0.51%) as compared with LTB-MUG broth (73%). Similar percentages were obtained if the results were calculated as geometric means; recovery rates of 0.52 and 61% were obtained for VRB-2 and LTB-MUG, respectively.

TABLE 3. Results of food and milk analyses by using the LTB-MUG-MPN method

Sample	LTB-MUG reaction[a]	
	Gas (%)	Fluorescence (%)[b]
Ground beef	3.9×10^3 (100)	1.1×10^3 (28)
Chicken giblets	4.5×10^2 (100)	5.6×10^1 (12)
Raw milk	7.8×10^2 (100)	2.0×10^1 (2.5)
Frozen broccoli	5.2×10^2 (100)	2.0×10^0 (0.4)

[a] Results are the average of three replicates.
[b] Percentage represents the fraction of fecal coliforms present in each food category.

In the chlorination study, the bacterial counts obtained on TGEA and VRB-2 agar before treatment were almost identical (Table 6). An arithmetic mean recovery rate of 95% was obtained on VRB-2 agar in relation to TGEA. After exposure of *E. coli* to 0.5 mg of chlorine per liter, a 99.9% mean reduction in viable cells was determined by the difference in counts on TGEA before and after treatment. The extent of chlorine-injured cells, as determined by the difference in counts on TGEA and VRB-2 agar, ranged from 87 to 89% for the three samples (Table 6). From the postexposure arithmetic mean recovery data, it is evident that neither VRB-2 agar nor LTB-MUG broth was efficient in detecting chlorine-injured cells. The LTB-MUG medium detected 31% of the cells in relation to TGEA, and the VRB-2 agar recovered only 11%. Recovery percentages based on geometric means were within 1% of the arithmetic mean results. The bacterial counts obtained on LTB-MUG were based on fluorescence; however, all tubes that showed fluorescence also showed gas production.

Bacterial interference. The presence of large numbers of *P. vulgaris* cells in LTB-MUG tubes effectively suppressed gas production by *E. coli* (false-negative MPN reactions); however, fluorescence due to *E. coli* GUD activity appeared within 15 h. Thus, *E. coli* could be detected in the presence of an antagonist that suppressed

TABLE 4. Results of EC broth fecal coliform confirmatory tests on LTB-MUG tubes obtained from the MPN analysis of food and milk samples[a]

Sample	LTB-MUG reaction	No. tested	Positive EC broth test (%)
Ground beef	Gas (+) fluorescence (+)	63	63 (100)
	Gas (+) fluorescence (−)	22	0 (0)
Chicken giblets	Gas (+) fluorescence (+)	97	86 (89)
	Gas (+) fluorescence (−)	4	0 (0)
Raw milk	Gas (+) fluorescence (+)	33	27 (82)
	Gas (+) fluorescence (−)	55	3 (5)
Frozen broccoli	Gas (+) fluorescence (+)	3	1 (34)
	Gas (+) fluorescence (−)	83	0 (0)

[a] Of 196 gas (+) fluorescence (+) samples, 177 (91%) had positive EC broth tests. Of 164 gas (+) fluorescence (−) samples, 3 (2%) had positive EC broth tests.

gas production (data not shown). Approximately 10^4 antagonistic cells to 1 *E. coli* cell per ml were required to suppress gas production. Pure cultures of *E. coli* that were inoculated into LTB-MUG tubes as controls produced both gas and fluorescence, but neither was detected in the *P. vulgaris* controls.

Rapid *E. coli* detection assay. A microtitration plate assay was developed for *E. coli* based solely on the criterion of fluorescence. As determined previously, fluorescence caused by GUD activity appeared before gas production from the fermentation of lactose. A five-well MPN assay with TSA-MUG medium was tested. It was found that with relatively contaminated samples such as sewage effluents, positive confirmation (fluorescence) could be obtained within 4 h (Fig. 5). Less contaminated samples required overnight incubation.

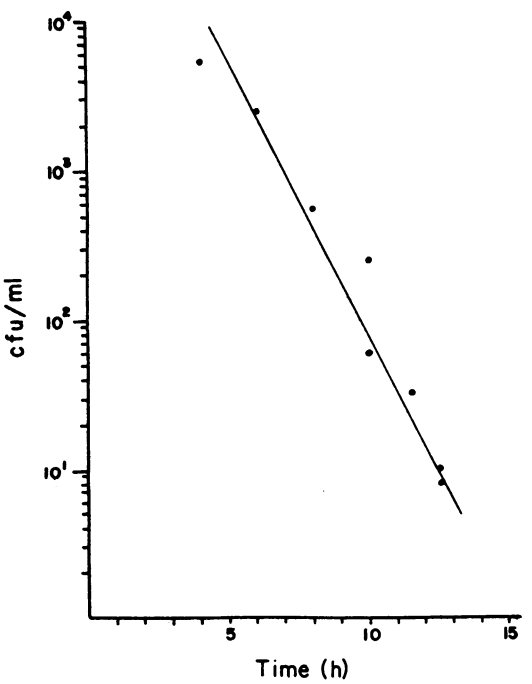

FIG. 4. Relationship between the initial cell count and the time that fluorescence occurred in LTB-MUG medium.

DISCUSSION

The presence of GUD in bacteria has been examined by several workers. Kilian and Bülow (26) tested 633 *Enterobacteriaceae* strains and reported that 97% of *E. coli* and 50% of *Shigella* spp. were the only ones positive for GUD. Those figures correlated well with results of this study; 96 and 40% of the *E. coli* and *Shigella* spp., respectively, produced this enzyme. LeMinor (28) examined over 4,000 salmonellae and found that approximately 30% produced GUD. Although the estimate of 17% obtained for *Salmonella* spp. in our study is lower than that reported by LeMinor (28), this was not unusual because GUD production in salmonellae is serotype specific (28).

Ten strains of enterotoxigenic *E. coli* that were examined were also positive for GUD activity. Enterotoxigenic and enteropathogenic strains of *E. coli* have been troublesome to detect and enumerate with standard procedures because some of these pathotypes were unable to ferment lactose, were sensitive to elevated temperatures, or were inhibited by routinely used enteric media (3, 35). It seems, therefore, that the fluorogenic GUD assay may be suitable as an initial screening method for the presumptive detection of *E. coli* pathotypes.

Other organisms, such as *Aeromonas* spp., *Enterobacter agglomerans*, and *Vibrio* spp., which have been reported to be easily mistaken for *E. coli* in routine analysis (27, 36, 41), could also be distinguished from *E. coli* by the fluorogenic GUD assay. We tested 4 *E. agglomerans* strains, and Kilian and Bülow (26) examined over 40 strains of *Vibrio* spp. and *Aeromonas*

TABLE 5. Comparative efficiency of the LTB-MUG-MPN method versus the VRB-2 method for the recovery of heat-injured *E. coli*

Sample	Recovery		
	TSA (%)	VRB-2 (%)	LTB-MUG (%)
1	4.4×10^7 (100)	5.3×10^4 (0.12)	3.4×10^7 (77)
2	4.5×10^6 (100)	3.5×10^4 (0.78)	1.9×10^6 (43)
3	1.6×10^7 (100)	2.4×10^5 (1.50)	1.1×10^7 (69)
Arithmetic mean	2.15×10^7 (100)	1.1×10^5 (0.51)	1.56×10^7 (73)
Geometric mean	1.46×10^7 (100)	7.6×10^4 (0.52)	8.92×10^6 (61)

spp.; none of these produced GUD. Although the production of GUD was not absolutely specific for *E. coli*, the fact that strains of *Salmonella* and *Shigella* also produced this enzyme would not significantly alter the sensitivity of the assay or the purpose for which these assays were intended.

The possibility of incorporating MUG directly into conventional coliform assays was tested. This idea was appealing because the efficiencies of these methods have already been established. Furthermore, coliform media were commonly used, commercially produced, and therefore, readily available.

In the membrane filter-mEndo broth and the VRB-2 direct plating methods, *E. coli* colonies were readily distinguished from the other coliforms by the appearance of fluorescent colonies or halos surrounding the colonies. This enabled a total coliform count (based on acid production from lactose) and a specific fecal coliform count (fluorescence) to be made on the same plate or membrane in a one-step procedure. Similarly, by using the LTB-MUG medium in an MPN assay, a presumptive coliform count could be obtained based on gas formation, and an immediate confirmation for *E. coli* could be obtained based on fluorescence. The presence of anaerogenic or weakly aerogenic strains of *E. coli* in LTB-MUG broth was also detected by the appearance of fluorescence. Non-gas-forming strains comprise about 5% of the *E. coli* population (12), and they are a cause of false-negative reactions in the presumptive, confirmed, and even completed MPN analyses (4, 43). In essence, by incorporating MUG in coliform assays, *E. coli* may be detected immediately in one-step procedures. Additional confirmatory tests or biochemical reactions are not necessary, which means considerable savings in labor, time, and media. In fact, if only an MPN for *E. coli* is desired, Durham tubes can be omitted from LTB-MUG broth and the tubes can be examined only for fluorescence.

The analysis of water samples showed that the fluorogenic LTB-MUG-MPN assay for *E. coli* was more efficient than the membrane filter-mFC broth assay for the enumeration of fecal coliforms. Since the incubation temperature of 45°C recommended for the mFC procedure is detrimental to the growth of some *E. coli* strains (34, 40), this method recovers fewer fecal coliforms than do MPN methods (6, 11, 29). The EC broth test for fecal coliforms confirmed that approximately 85% of the gas (+) fluorescence (+) tubes contained *E. coli*. A 9% apparently false-positive rate was also observed. These results were not unusual because Geldreich (16) reported that about 90% of *E. coli* and 8% of nonfecal coliforms produced gas in the EC broth test. The most interesting aspect, however, was that 17 gas (−) fluorescence (+) tubes failed to produce gas in the EC broth confirmatory test. Of these 17 apparently false-negative tubes, 2 anaerogenic strains of *E. coli*, 1 *Salmonella* sp., and 1 *Shigella* sp. accounted for the appearance

TABLE 6. Comparative efficiency of the LTB-MUG-MPN method versus the VRB-2 method for the recovery of chlorine-injured *E. coli*

Sample	Recovery				
	Untreated		Treated[a]		
	TGEA (%)	VRB-2 (%)	TGEA (%)	VRB-2 (%)	LTB-MUG (%)
1	2.28×10^7 (100)	2.08×10^7 (91)	8.2×10^3 (100)	9.0×10^2 (11)	2.6×10^3 (32)
2	1.96×10^7 (100)	1.94×10^7 (99)	1.0×10^3 (100)	1.1×10^2 (11)	2.3×10^2 (23)
3	1.65×10^7 (100)	1.56×10^7 (95)	1.0×10^2 (100)	1.3×10^1 (13)	3.5×10^1 (35)
Arithmetic mean	1.96×10^7 (100)	1.86×10^7 (95)	3.1×10^3 (100)	3.4×10^2 (11)	9.6×10^2 (31)
Geometric mean	1.94×10^7 (100)	1.84×10^7 (95)	9.4×10^2 (100)	1.1×10^2 (12)	2.8×10^2 (30)

[a] Treated with 0.5 mg of chlorine per liter for 8 min at 25°C.

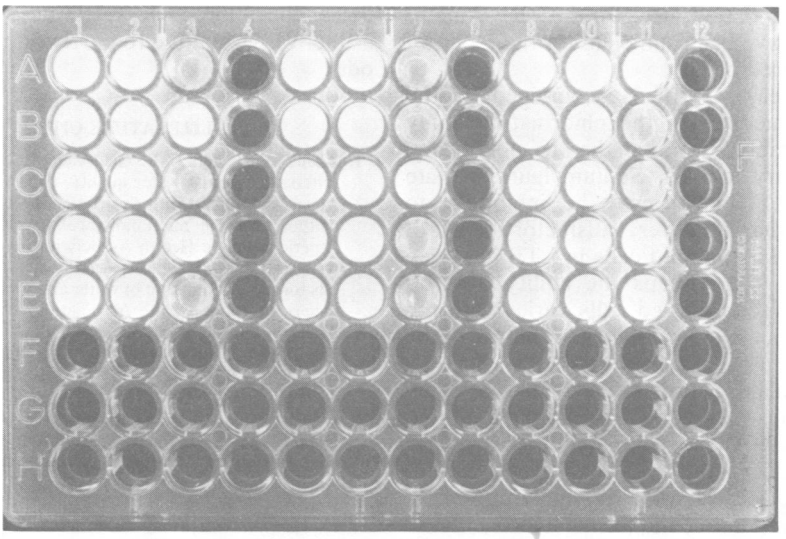

FIG. 5. Appearance of a rapid five-well MPN analysis with microtitration plates filled with TSA-MUG medium. Wells 1 through 3, 5 through 7, and 9 through 11 are replicate five-well assays; the results (left to right) are 5+, 5+, 2+; 5+, 5+, 1+; and 5+, 5+, 3+, respectively.

of fluorescence. With regard to the other 13 tubes, strains of *E. coli* might have been present but were not isolated by the procedures that we used, possibly because of coliform suppression. The masking of coliforms is prevalent in waters with high bacterial densities (44). Although we did not observe a correlation between high total background count and incidences of gas (−) fluorescence (+) reactions (data not shown), the lack of correlation did not rule out the possibility that interference might have occurred. Coliform suppression due to the production of bacteriocin-like substances by antagonists has been reported (46).

In milk and food samples, approximately 91% of all gas (+) fluorescence (+) tubes were confirmed to contain *E. coli* by the EC broth test. A low false-positive rate of 2% was also observed. These percentages were in good agreement with the results obtained by Geldreich (16), and the rate of false-positive reactions was even lower than the 8% reported by him.

The sensitivity of the LTB-MUG medium was similar to those of impedimetric assays (7, 42). The presence of 1 viable cell was detected within 20 h, and approximately 10^7 to 10^8 cells per ml were required to produce fluorescence. Impedance assays often require a threshold concentration of 10^6 to 10^7 cells per ml before conductivity changes can be detected. The results of the fluorogenic test were available much faster than conventional methods that rely upon the detection of gas evolution.

Factors such as heating, freezing, drying, or the presence of a toxic chemical such as chlorine can cause injury to cells (5, 8, 22). Debilitated bacteria have the ability to recuperate and grow on nonselective media; therefore, they are troublesome to enumerate with routine coliform analysis procedures. The LTB-MUG medium was superior to the VRB-2 agar overlay method for the recovery of heat-injured *E. coli* cells. Mean recoveries of 71 and 0.51% were obtained for LTB-MUG and VRB-2 medium, respectively, in relation to recoveries on TSA. VRB agar has been reported to be selective, and thus inadequate, in recovering injured cells (39). However, in our study, the VRB-2 method (21) was used, which included a 2-h resuscitation period on TSA; therefore, the low recovery rate (0.51%) was not expected. Possibly, the highly selective nature of VRB agar was inhibitory to some of the *E. coli* cells. The occurrence of VRB-sensitive strains of *E. coli* has been reported (31).

Exposure of *E. coli* cells to 0.5 mg of chlorine per liter caused an injury rate of 89%. Camper and McFeters (8) used this same free chlorine concentration and reported an approximate cell injury rate of 90%. The recovery data from the chlorination studies showed that neither LTB-MUG nor VRB-2 medium was adequate; however, the LTB-MUG medium was almost three times more efficient than VRB-2 agar for enumerating chlorine-injured bacteria. The recovery rate of 11% obtained with VRB-2 agar was again unexpectedly low. Camper and McFeters (8) reported that chlorine-injured *E. coli* cells often show a 3-h lag period in selective media before growth initiation. Therefore, the 2-h

preenrichment that we used for the VRB-2 method may not have been sufficient for the repair of severely injured cells.

The recovery rate of 31% obtained by the LTB-MUG medium in the chlorination study was considerably lower than the 71% obtained in the heat injury studies. Sodium lauryl sulfate present in LTB broth may be inhibitory to injured cells (38); however, satisfactory repair of heat and chlorine injured cells in LTB has been reported (9, 40). Perhaps the ability of LTB medium to rescue injured cells varies with the severity of injury, type of injury, or nature of the treatment.

Injured cells sometimes are defective in enzyme activities (22). Green and Stumpf (19) and McKee et al. (32) believed that chlorine damages cells by oxidizing the sulfhydryl groups of enzymes. Aldolase, hexokinase, glucose-6-phosphate dehydrogenase, and formic hydrogenlyase have all been speculated to be affected (32, 37, 45). However, the tubes of LTB-MUG medium that we inoculated with heat- or chlorine-injured *E. coli* cells and that became fluorescent also contained gas. This suggested that neither GUD nor formic hydrogenlyase was damaged, unless both enzymes were affected at the same time.

The occurrence of bacterial interference in lactose broth and in LTB medium has been well documented. Braswell and Hoadley (6) and Olson (37) reported that coliforms often fail to produce gas in the presence of antagonistic bacteria, resulting in false-negative presumptive MPN tests. It was determined that an approximate *E. coli*-to-*P. vulgaris* ratio of 1:10,000 in LTB-MUG tubes suppressed gas production by *E. coli*; however, *E. coli* was effectively detected by the appearance of fluorescence. Hutchinson et al. (25) reported that suppression of *E. coli* occurs most often when the antagonists are present in a density range of 10,000 cells per ml. Incidences of coliform suppression by *P. vulgaris* have been reported by others (17, 18, 25).

In conclusion, it seems that fluorogenic assays are applicable to routine coliform analyses. By incorporating MUG into conventional coliform assays, the ability to detect *E. coli* is improved, and the amount of time, media, and labor required for each analysis is reduced. Studies with bacterial interference and injured cells establish that the fluorogenic test has definite advantages over conventional assays. Furthermore, the tests are efficient and accurate for the analysis of naturally contaminated samples. The fluorogenic test may also be adapted to rapidly screen and enumerate *E. coli*. These tests are amenable to automation. By eliminating the battery of biochemical tests and the laborious manipulations now required for *E. coli* identification and yet providing simplicity, sensitivity, and specificity, the fluorogenic GUD assay may be an attractive alternative to current detection methods.

LITERATURE CITED

1. **Allen, M. J., and E. E. Geldreich.** 1975. Bacteriological criteria for ground-water quality. Ground Water **13**:1–7.
2. **American Public Health Association.** 1976. Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D.C.
3. **American Public Health Association.** 1981. Standard methods for the examination of water and wastewater, 15th ed. American Public Health Association, Washington, D.C.
4. **Anderson, J. G., P. S. Meadows, B. W. Mullins, and K. Patel.** 1980. Gas production by *Escherichia coli* in selective lactose fermentation media. FEMS Microbiol. Lett. **8**:17–21.
5. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. **29**:186–194.
6. **Braswell, J. R., and A. W. Hoadley.** 1974. Recovery of *Escherichia coli* from chlorinated secondary sewage. Appl. Microbiol. **28**:328–329.
7. **Cady, P., S. W. Dufour, J. Shaw, and S. J. Kraeger.** 1978. Electrical impedance measurements: rapid method for detecting and monitoring microorganisms. J. Clin. Microbiol. **7**:265–272.
8. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
9. **Clark, C. W., and Z. J. Ordal.** 1969. Thermal injury and recovery of *Salmonella typhimurium* and its effect on enumeration procedures. Appl. Microbiol. **18**:332–336.
10. **Dahlén, G., and A. Linde.** 1973. Screening plate method for detection of bacterial β-glucuronidase. Appl. Microbiol. **26**:863–866.
11. **Dutka, B. J., S. Kuchma, and K. K. Kwan.** 1979. Fecal coliform and *E. coli* estimates, tip of the iceberg. Water Air Soil Pollut. **1979**:349–362.
12. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of the *Enterobacteriaceae*, 3rd ed. Burgess Publishing Co., Minneapolis.
13. **Evans, T. M., M. W. LeChevallier, C. E. Waarvick, and R. J. Seidler.** 1981. Coliform species recovered from untreated surface water and drinking water by membrane filter, standard, and modified most-probable-number techniques. Appl. Environ. Microbiol. **41**:657–663.
14. **Evans, T. M., C. E. Waarvick, R. J. Seidler, and M. W. LeChevallier.** 1981. Failure of the most-probable-number technique to detect coliforms in drinking water and raw water supplies. Appl. Environ. Microbiol. **41**:130–138.
15. **Fishman, W. H., B. Springer, and R. Burnett.** 1948. Application of an improved glucuronidase assay method to the study of human blood. J. Biol. Chem. **173**:449–455.
16. **Geldreich, E. E.** 1966. Sanitary significance of fecal coliforms in the environment. Water Pollution Research publication no. WP-20-3. U.S. Department of the Interior, Washington, D.C.
17. **Geldreich, E. E.** 1973. Is the total count necessary? Proc. Am. Water Works Assoc. Water Qual. Technol. Conf. **7**:1–11.
18. **Geldreich, E. E., H. D. Nash, D. J. Reasoner, and R. H. Taylor.** 1972. The necessity of controlling bacterial populations in potable water: community water supply. J. Am. Water Works Assoc. **64**:596–602.
19. **Green, D. E., and P. K. Stumpf.** 1946. The mode of action of chlorine. J. Am. Water Works Assoc. **38**:1301–1305.
20. **Greer, F. E., and F. V. Nyhan.** 1928. The sanitary significance of lactose fermenting organisms not belonging to the *B. coli* group. 3. Bacterial associations in cultures containing lactose fermenting bacteria. J. Infect. Dis. **42**:525–536.

21. **Hartman, P. A., P. S. Hartman, and W. W. Lanz.** 1975. Violet red bile 2 agar for stressed coliforms. Appl. Microbiol. **29**:537–539. (Erratum **29**:865.)
22. **Hurst, A.** 1977. Bacterial injury: a review. Can. J. Microbiol. **23**:935–944.
23. **Hussong, D., R. R. Colwell, and R. M. Weiner.** 1980. Rate of occurrence of false-positive results from total coliform most-probable-number analysis of shellfish and estuaries. Appl. Environ. Microbiol. **40**:981–983.
24. **Hussong, D., J. M. Damaré, R. M. Weiner, and R. R. Colwell.** 1981. Bacteria associated with false-positive most-probable-number coliform test results for shellfish and estuaries. Appl. Environ. Microbiol. **41**:35–45.
25. **Hutchinson, D., R. H. Weaver, and M. Scherago.** 1943. The incidence and significance of microorganisms antagonistic to *Escherichia coli* in water. J. Bacteriol. **45**:29–34.
26. **Kilian, M., and P. Bülow.** 1976. Rapid diagnosis of *Enterobacteriaceae*. I. Detection of bacterial glycosidases. Acta Pathol. Microbiol. Scand. Sect. B **84**:245–251.
27. **LeChevallier, M. W., R. J. Seidler, and T. M. Evans.** 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. Appl. Environ. Microbiol. **40**:922–930.
28. **LeMinor, L.** 1979. Tetrathionate reductase, β-glucuronidase, and ONPG-test in the genus *Salmonella*. Zentralbl. Bakteriol. Parasitenkd. Infekleonskr. Hyg. Abt. 1, Orig. Reihe A **243**:321–325.
29. **Lin, S.** 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollut. Control Fed. **45**:498–506.
30. **Maddocks, J. L., and M. J. Greenan.** 1975. A rapid method for identifying bacterial enzymes. J. Clin. Pathol. **28**:686–687.
31. **Maxcy, R. B.** 1973. Condition of coliform organisms influencing recovery of subcultures on selective media. J. Milk Food Technol. **36**:414–416.
32. **McKee, J. E., R. T. McLaughlin, and P. Lesgourgues.** 1958. Application of molecular filter techniques to the bacterial assay of sewage. III. Effect of physical and chemical disinfection. Sewage Ind. Wastes **30**:245–252.
33. **Mead, J. A. R., J. N. Smith, and R. T. Williams.** 1955. The biosynthesis of the glucuronides of umbelliferone and 4-methylumbelliferone and their use in fluorimetric determination of β-glucuronidase. Biochem. J. **61**:569–574.
34. **Meadows, P. S., J. G. Anderson, and K. Patel.** 1980. Synergistic inhibition of *Escherichia coli* growth and gas production in selective media. FEMS Microbiol. Lett. **8**:215–219.
35. **Mehlman, I. J., N. T. Simon, A. C. Sanders, M. Fishbein, J. C. Olson, Jr., and R. B. Read.** 1975. Methodology for enteropathogenic *Escherichia coli*. J. Assoc. Off. Anal. Chem. **58**:283–292.
36. **Neilson, A. H.** 1978. The occurrence of aeromonads in activated sludge: isolation of *Aeromonas sobria* and its possible confusion with *Escherichia coli*. J. Appl. Bacteriol. **44**:259–264.,
37. **Olson, B. H.** 1978. Enhanced accuracy of coliform testing in seawater by a modification of the most-probable-number method. Appl. Environ. Microbiol. **36**:438–444.
38. **Ray, B.** 1979. Methods to detect stressed microorganisms. J. Food Prot. **42**:346–355.
39. **Ray, B., and M. L. Speck.** 1973. Enumeration of *Escherichia coli* in frozen samples after recovery from injury. Appl. Microbiol. **25**:499–503.
40. **Rose, R. E., E. E. Geldreich, and W. Litsky.** 1975. Improved membrane filter method for fecal coliform analysis. Appl. Microbiol. **29**:532–536.
41. **Rosen, A., and R. E. Levin.** 1970. Vibrios from fish pen slime which mimic *Escherichia coli* on violet red bile agar. Appl. Microbiol. **20**:107–112.
42. **Silverman, M. P., and E. F. Munoz.** 1979. Automated electrical impedance technique for rapid enumeration of fecal coliforms in effluents from sewage treatment plants. Appl. Environ. Microbiol. **37**:521–526.
43. **Tham, W., and M.-L. Danielsson.** 1980. Reliability of VRB agar and BGLB broth for enumeration of 44°C coliforms in food. Nord. Veterinaermed. **32**:325–331.
44. **Thompson, R. E.** 1927. Irregularities in the test for *B. coli* in water. J. Bacteriol. **13**:209–221.
45. **Toth, D., and E. Karelova.** 1977. Interrelation between enzyme activity and ability of multiplying of *Escherichia coli* cells after exposure to deficient environment. Biologia **32**:399–408.
46. **Weaver, R. H., and T. Boiter.** 1951. Antibiotic-producing species of *Bacillus* from well water. Trans. Ky. Acad. Sci. **13**:183–188.